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Minireview

Beyond the epithelium: Cadherin function in fibrous connective tissues

T.Y. El Sayegh[a,*], A. Kapus[b], C.A. McCulloch[a]

[a] CIHR Group in Matrix Dynamics, University of Toronto, Room 243, Fitzgerald Building, 150 College Street, Toronto, Ont., Canada M5S 3E2
[b] Department of Surgery, University of Toronto and St. Michael's Hospital Research Institute, Toronto, Canada

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Abstract In fibrous connective tissues, fibroblasts are organized into syncytia, cellular networks that enable matrix remodeling and that are interconnected by intercellular adherens junctions (AJs). The AJs of fibroblasts are mediated by N-cadherin, a broadly expressed classical cadherin that is critically involved in developmental processes, wound healing and several diseases of mesenchymal tissues. In contrast to E-cadherin-dependent junctions of epithelia, the formation of AJs in fibrous connective tissues is relatively uncharacterized. Work over the last several years has documented an expanding list of molecules which function to regulate N-cadherin mediated junctions such as: Fer, PTP1B, cortactin, calcium, gelsolin, PIP5KIγ, PIP2, and the Rho family of GTPases. We present an overview on the regulation of N-cadherin-mediated junction formation that highlights recent molecular advances in the field and rationalizes the roles of N-cadherin in connective tissue function.

Keywords: Fer; Cortactin; N-cadherin; Actin; Adhesion strength; Lateral clustering

1. Adherens junctions in connective tissues

Despite a broader expression profile than E-cadherin, much less is known of N-cadherin-mediated adhesions with respect to developmental and pathological processes. While some of the central paradigms of cadherin biology derived from E-cadherin models may apply to the other classical cadherins, important differences of function and regulation between E and N-cadherin-mediated adherens junctions (AJs) are emerging that merit critical analysis.

AJs are adhesive plaques located at the junction of contacting cells and are mediated by the classical cadherins, their adapter proteins, and the actin microfilament network. AJs of epithelial cells are typically located subjacent to a zone of tight junctions at the interface between the apical and basal regions of the cell. In epithelial cells AJs are mediated by E-cadherin, the best characterized molecule in the cadherin superfamily. AJ-like structures can also be formed by N-cadherin [4,5] which is broadly expressed by cells in nervous, fibrous, mineralized, muscular, and adipose tissues. N-cadherin-mediated AJs are of central importance in connective tissue physiology and are critical for the regulation of cell attachment and migration [6], wound healing [9], metastatic potential [10], embryonic development [13,14], differentiation and formation of numerous specialized tissues including fibrous connective tissues [19–23]. N-cadherin may also play an important role in the formation of sarcomas [24,25] but much less is known of these lesions compared to E-cadherin and carcinomas.

While there are several reviews on the role of N-cadherin mediated AJs in bone and cartilage [23,26,27], nervous tissue [21,28], or muscle [22,29], there are no comprehensive reviews on fibrous connective tissues. This review integrates current understanding of the formation and regulation of N-cadherin-mediated AJs in fibrous connective tissues of vertebrates along with exposition of their physiological significance.

2. Adherens junction morphology

While fibroblasts express several different cadherins including P-cadherin, R-cadherin (cadherin-4), OB-cadherin (cadherin-11), and fat-like cadherins [30,31], N-cadherin is the predominant cadherin expressed by these cells (see Text Box 1) [30,32]. Ultrastructural studies show that fibroblasts form AJ structures that exhibit similar structural organization as epithelial cells [4,5,33,34]. Proteins found in epithelial AJs such as β-catenin, p120 catenin, α-catenin and vinculin also co-localize to regions of intercellular contact or to regions of cadherin binding in fibroblasts [4,33,35,36]. Actin filaments associate with these AJs perpendicular to the membrane but without an underlying radial actin belt that is typical of epithelial cells [33,36].

*Corresponding author. Fax: +1 416 978 5956.
E-mail address: t.elsayegh@utoronto.ca (T.Y. El Sayegh).

Abbreviations: AJ, adherens junctions; WASP, Wiskott–Aldrich syndrome protein; PIP5KIγ, phosphatidylinositol phosphate 5 kinase; PIP2, phosphatidylinositol 4,5 bisphosphate; Arp2/3, actin related protein 2/3; Ca^{2+}, calcium ions

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Text Box. Regulation of N-cadherin expression and surface transport [1–3,7,8,11,12,15–18]

The human N-cadherin gene consists of 16 exons and is located in a 250-kb region on chromosome 18q11.2 [1]. Investigation of the chicken N-cadherin promoter revealed no CATT or TATA boxes [2]. Later studies described an NFκB transcription factor binding site and demonstrated that N-cadherin expression may be directly regulated by NFκB activation [3]. Following transcription, N-cadherin molecules undergo glycosylation and processing in the endoplasmic reticulum and golgi apparatus followed by association with catenin molecules prior to exocytic trafficking and function on the cell surface [7,8]. Unlike E-cadherin, the core catenin proteins complex with pro-N-cadherin molecules at a much earlier stage in synthesis starting with the binding of p120 catenin [11,12]. β and α-catenin molecules bind to pro-N-cadherin molecules subsequent to direct phosphorylation of the cytoplasmic domain (possibly by casein kinase II) and prior to furin mediated propeptide cleavage [12,15,16]. The binding of the catenin molecules is necessary for the appropriate transport of the N-cadherin molecule to the cell surface and for engagement into AJs [17,18]. Surface trafficking of N-cadherin is dependent on an intact microtubule network as well as on the motor function of kinesin, a microtubule associated motor protein, whose heavy chains bind to p120 catenin [17,18].

Ultrastructural studies of the periodontal ligament, a highly cellular and vascular, fibrous connective tissue have revealed closely appositioned fibroblasts with numerous gap and AJs [34,37]. AJs were macular in shape, had a diameter of 0.1–0.4 μm, an average length of 172 nm and were in close proximity to gap junctions, with membranes separated by a space of 10–23 nm, [34]. The majority of close, inter-membrane appositions between cells were AJs; each fibroblast exhibited ~20–30 AJs and 5–20 gap junctions, depending on the anatomical location of the tissue sections [34]. This extensive network of AJs in connective tissue fibroblasts provides the structural foundation for the intercellular collaboration necessary for extracellular matrix turnover and for mechanical coupling of cells in connective tissues undergoing turnover or subject to mechanical loading [20,38].

3. Adhesion strength

A critical question in cadherin biology is how adhesion strength is regulated. This is an important determinant of the formation and dissolution of intercellular contacts and is also of relevance to metastasis, a process in which tumor cells lose their intercellular adhesions and acquire a more invasive and migratory phenotype [39]. N-cadherin-dependent AJs demonstrate intrinsically weaker adhesion strength than E-cadherin-dependent AJs [40,41]. This discrepancy may be related to variations in the organization of the subcortical actin network (see Section 2), or to the differential recruitment of cadherin-associated proteins to AJs. Indeed, when expressed in a cadherin-null pancreatic carcinoma cell line, N-cadherin and E-cadherin bind different isoforms of p120 catenin: N-cadherin binds a larger phosphorylated isoform of p120 while E-cadherin binds a smaller, non-phosphorylated isoform [42]. As p120 catenin and the extent of phosphorylation have been implicated in the regulation of cadherin adhesive strength [43], variations of isoform binding to E and N-cadherin may explain some of the differences of adhesion strength [40]. Indeed, longer splice variants of p120 catenin are expressed in mesenchymal cell tumors and are associated with reduced adhesion strength [43–45]. Collectively these findings suggest that intrinsic differences between E and N cadherins play important roles in regulating cellular phenotype and cadherin adhesion strength in connective tissue, epithelial and transformed cells.

The extracellular domain of type I cadherins displays weak intrinsic binding activity, and the conversion to strong intercellular adhesion requires the presence of the cytoplasmic domain and adapter proteins [46]. The precise mechanism by which weak initial adhesions are transformed into strong contacts is not defined. While alterations that lead to increased single cadherin bond strength (affinity of homophilic trans interactions) have not been explored, there is considerable evidence to suggest that increases in cadherin avidity are important for contact strengthening. Accordingly, the lateral clustering of surface-expressed cadherins is recognized as a fundamental determinant of cadherin mediated contact formation [47,48]. These variations of cadherin avidity are dependent on the integrity of the cadherin cytoplasmic domain, associated binding proteins and related signaling networks [48,49].

4. Cadherin dynamics

Comparisons between the dynamics of AJs formed in fibroblasts and epithelial cells in vitro demonstrate fundamentally different kinetics and maturation of contacts. Adjacent non-transformed fibroblasts make initial contacts, which then progress to a superimposition or overlap of contacting lamellipodia [36]. This is typically followed by retraction of contacting membranes, loss of contact and extension of cellular protrusions and directed cellular movement away from the former site of contact [36]. In contrast, adjacent non-transformed epithelial cells form intercellular contacts that expand laterally, resulting in broad zones of contact along the circumference of the cell periphery [36,50] (Fig. 1). More detailed observations on the progression and localization of cadherins at sites of intercellular contact have used live cell imaging of GFP-tagged, full-length cadherin constructs during contact formation and maturation [48,51]. While most intercellular adhesions in cultured fibroblasts are short-lived, some N-cadherin-mediated junctions are quite stable over prolonged periods. After formation, these junctions mature by lengthening of the contact area in a similar fashion to that observed in epithelial cells [48]. These results demonstrate that N-cadherin may independently, or in conjunction with other intercellular adhesion molecules [52,53], mediate long-lasting contacts in mesenchymal cells that are required for proper organ/tissue function and intercellular communication [4,14,54].

5. Formation and maturation of adherens junctions in fibroblasts

In mechanically loaded, rapidly remodeling fibrous connective tissues such as the periodontal ligament, fibroblasts exhibit an extensive network of AJs that provide direct physical coupling between adjacent cells [34,37,55]. Cadherin-mediated

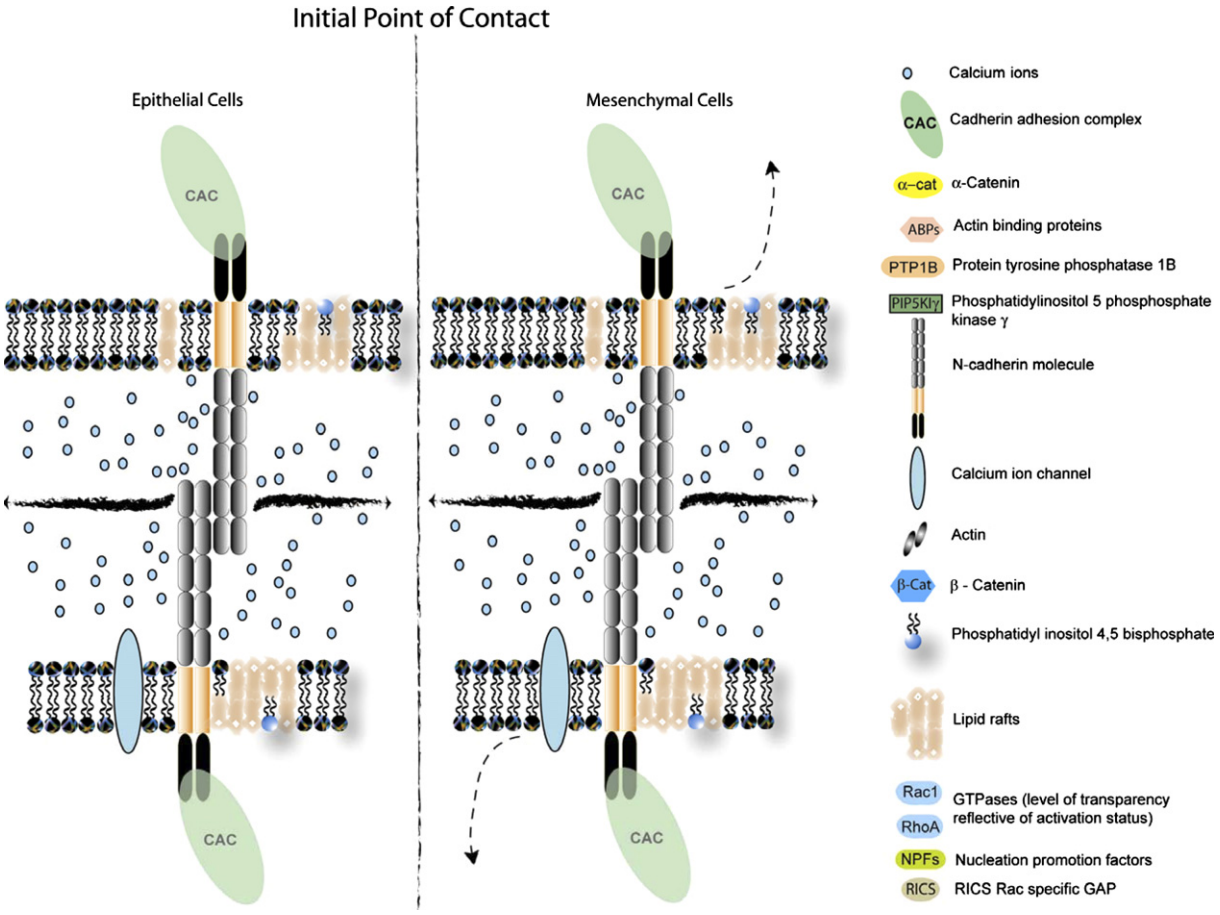

Fig. 1. Initial E-cadherin contacts in epithelial cells are more stable than N-cadherin contacts in mesenchymal cells and a more likely to mature forming broad belt-like zones of adhesion leading to AJ stability. Thick arrows denote contact zone extension and dashed arrows remodeling of initial contacts. Legend indicates molecules used in this and subsequent diagrams.

AJs in connective tissue fibroblasts are important for physiological turnover of the extracellular matrix, wound contraction and intercellular mechanotransduction [55,56]. While the formation and maturation of N-cadherin-mediated AJs are still poorly characterized, recent advances suggest plausible regulatory mechanisms.

AJ formation in fibroblasts is initiated by random collisions between cellular extensions of adjacent cells. Upon contact the first two cadherin repeats of the extracellular domain of N-cadherin molecules from opposing cells engage and produce a trans dimer-mediated initial adhesion [57] (Fig. 1). Sustained contact requires adhesion complex formation, lateral clustering of N-cadherin molecules, actin remodeling, and contact zone extension. These processes are coordinated by a complex array of signaling and regulatory pathways.

5.1. Recruitment of the cadherin adhesion complex and activation of the Rho GTPases

Following engagement of N-cadherin molecules from opposing cell surfaces, several proteins including α, β and p120 catenins, Fer, cortactin, nonreceptor tyrosine phosphatase PTP1B, gelsolin, and PIP5KIγ, and actin nucleators (e.g. Wiskott–Aldrich syndrome protein [WASP]) are recruited to the cadherin adhesion complex [48,58–63] (Fig. 2: early N-cadherin ligation). While molecules such as Fer are constitutively bound to catenins [62], others (e.g. gelsolin) are recruited by unknown signaling pathways [60]. The recruitment of cortactin and PIP5KIγ is dependent on the activation and signaling events involving distinct members of Rho family GTPases [61] (Fig. 2: early N-cadherin ligation).

Activation of Rho GTPases following engagement of type I cadherins is an early event in AJ formation [64]. The precise mechanism of how cadherin ligation results in activation of the Rho GTPases is unknown (Fig. 2, black star: early N-cadherin ligation). Consistent with studies in myoblasts [65], RhoA activation is detected after N-cadherin ligation in fibroblasts. However in fibroblasts, Rac1 activation precedes RhoA activation and occurs transiently during the first 30 min of attachment to N-cadherin-coated substrata [48]. The overlap in activation of Rac1 and RhoA during N-cadherin-mediated contact formation and maturation is minimal, in agreement with previous studies demonstrating a reciprocal balance between these GTPases [66]. Indeed unidirectional inhibition from Rac1 to RhoA in 3T3 cells relying on a mechanism independent of the distinct cytoskeletal remodeling events induced by Rac1 has been previously noted [66] (Fig. 2: early N-cadherin ligation). Rac1 activation correlates temporally with events required for the formation of early N-cadherin-mediated contacts. These events include contact zone extension of lamellipodia and the recruitment of actin to sites of N-cadherin ligation [67,68]. Rac1 activation is also required for the recruitment of cortactin to sites of nascent N-cadherin ligation

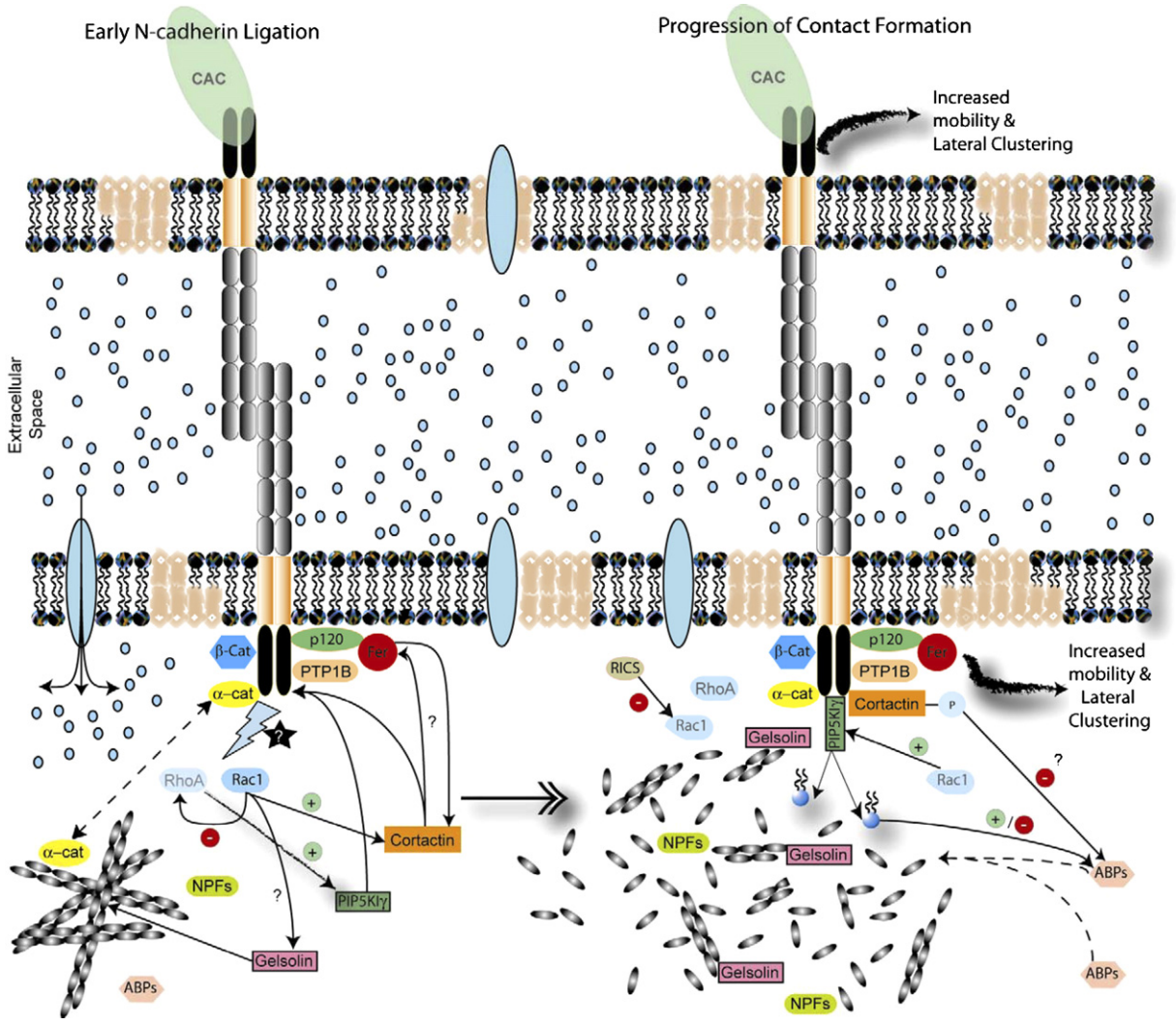

Fig. 2. Early events after N-cadherin ligation detailed to the left of the diagram. Recruitment of numerous actin binding proteins including cortactin and gelsolin, activation of Rho family of GTPases by undetermined mechanisms (black star) and transient intracellular calcium ion flux. Ca^{2+} influx activates gelsolin-mediated actin severing. Recruitment of actin binding proteins to sites of N-cadherin ligation facilitates remodeling of cortical actin cytoskeleton. Events leading to the progression of N-cadherin-mediated AJ formation detailed to the right of the diagram. Fer-mediated cortactin phosphorylation increases lateral mobility of N-cadherin, facilitates lateral clustering, and maturation of AJs. Rac stimulated phosphatidylinositol 5 phosphate kinase γ (PIP5KIγ) catalyzes formation of PIP2, which regulates gelsolin and the function of other actin binding proteins.

[48], the activation of PIP5KIγ-mediated PIP2 synthesis, and gelsolin-mediated uncapping of barbed actin filaments [69] (Fig. 2: entire diagram).

Thus the spatiotemporal regulation of Rho GTPase signaling events at AJs is unfolding as an integral component of AJ maturation. While the molecular mediators that regulate the activation profile of the Rho GTPases remain largely unknown, inhibition of Rac activation by the novel GAP RICS is an attractive possibility [70] (Fig. 2: progression of contact formation). These processes may lead to the release of inhibitory constraints on RhoA activation and associated signaling events (e.g. ROCK signaling) which manifest during AJ maturation [48,71].

5.2. Calcium as a regulator of gelsolin and N-cadherin AJ formation

An early event after N-cadherin ligation is the activation of plasma membrane stretch-sensitive Ca^{2+} permeable channels and transient increases of Ca^{2+} subjacent to sites of intercellular contact [55] (Fig. 2: early N-cadherin ligation). In connective tissue fibroblasts these transients are important determinants of cell contractility, motility [72,73] and the formation of intercellular adhesions [55,74]. Upon N-cadherin ligation, the amplitude of juxtamembrane Ca^{2+} fluxes correlate temporally with the recruitment of actin assembly at regions of cadherin-mediated adhesions [55,74]. Intracellular Ca^{2+} fluxes are also required for filopodial protrusions enriched with vinculin, VASP, mena and zyxin; these proteins are necessary for AJ formation by increasing interdigitation between membranes of adjacent cells [75]. While the proteins that mediate these Ca^{2+}-dependent filopodial propulsions and the underlying actin polymerization are not defined, gelsolin is a possible candidate [60].

The actin filament severing activity of gelsolin is regulated by free Ca^{2+} concentration. Blockade of plasma membrane-associated Ca^{2+} channels reduces the adhesion strength of gelsolin wild type cells but without influencing the strength of gelsolin null cells [60]. Lack of gelsolin expression results in a poorly

developed subcortical actin architecture which may be related to the requirement of gelsolin actin severing activity for actin related protein 2/3 (Arp2/3) dependent actin polymerization [60,76]. The resultant altered actin architecture negatively impacts on N-cadherin-mediated contact formation and adhesion strength [60]. Previous studies have documented the importance of gelsolin as an important downstream effector of Rac1-mediated cytoskeletal dynamics in fibroblasts [77]. While the precise mechanism of Rac1-mediated regulation of gelsolin has not been determined, it may involve the dissociation of gelsolin from actin filaments resulting in uncapping of polymerization-competent barbed ends [69] (Fig. 2: early N-cadherin ligation).

Calcium signaling has been cited in the regulation of the translocation and activation of the small GTPase Rac1 [78,79]. Cadherin-stimulated Ca^{2+} transients could provide a mechanism to locally recruit Rac1 to sites of N-cadherin ligation. Further, as the Rac/Cdc42-specific GAP RICS is negatively regulated by the Ca^{2+}/calmodulin-dependent protein kinase II [70], cessation of Ca^{2+} flux may trigger activation of RICS and inhibition of Rac1 (Fig. 2: progression of contact formation). Collectively, these findings suggest a central role for Ca^{2+} transients in regulating N-cadherin function by regulating Rac1 translocation and signaling events.

5.3. Lateral clustering and the role of Fer

The juxtamembrane domain of type I cadherins and specifically p120 catenin, are necessary for lateral clustering of cadherins, a key determinant of AJ strength and maturation [47,49]. While the molecular determinants and mechanisms of this process are not characterized [49], the non-receptor tyrosine kinase Fer and its substrate cortactin are implicated [48]. Cortactin is transiently recruited to nascent AJs in a Rac1-dependent mechanism. Cortactin then physically associates with the N-cadherin adhesion complex at nascent contacts and is transiently phosphorylated by p120 catenin-associated

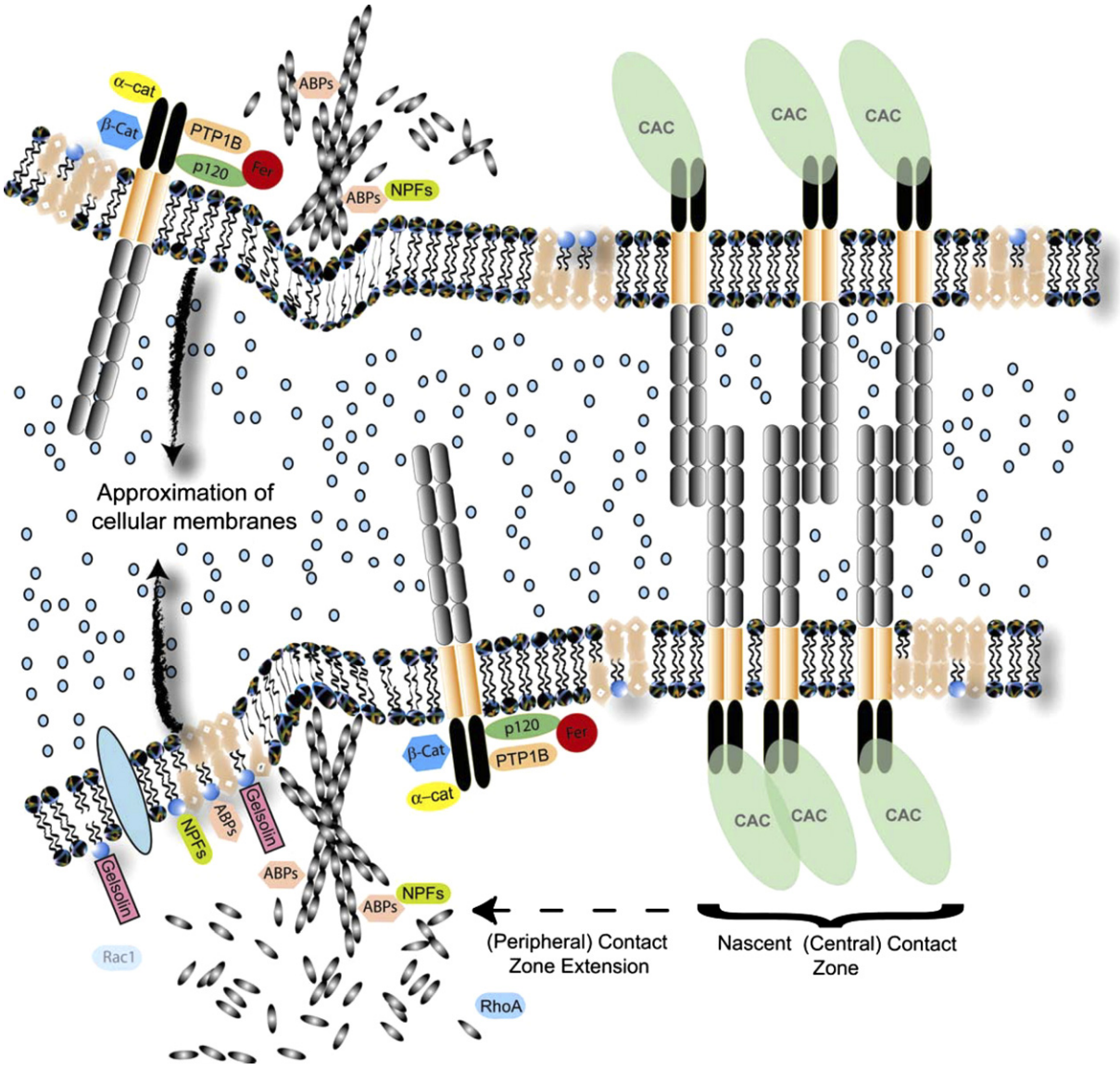

Fig. 3. Maturation of contacts and zone extension. Lateral mobility creates distinct zones of adhesion. Locally regulated actin polymerization results in reorganization of the cytoskeletal network, membrane protrusions, engagement of increased number of cell surface expressed N-cadherin molecules and contact zone extension.

Fer [48,61] (Fig. 2). Tyrosine phosphorylation of cortactin is necessary for increased lateral mobility and lateral clustering of surface-expressed N-cadherin [48] (Fig. 2). The reduced cortactin tyrosine phosphorylation of fibroblasts derived from fer kinase knockout mice inhibits surface cadherin mobility and ligand-induced clustering [48].

How tyrosine phosphorylation of cortactin enhances the mobility of surface-expressed N-cadherin is not known. The primary constraint for cadherin mobility may be restrictive cortical actin filament configurations, consistent with the membrane-cytoskeleton fence model [80,81]. Notably, cortactin tyrosine phosphorylation occurs at sites of actin depolymerization [82]. Tyrosine phosphorylation of cortactin also reduces N-WASP and WASP-mediated activation, which may impact on the regulation of de novo actin polymerization by the Arp2/3 complex [83]. Thus in conjunction with localized gelsolin-mediated actin severing activity, cortactin tyrosine phosphorylation at nascent AJs may be important for regulating the reorganization of cortical actin networks necessary to liberate N-cadherin molecules from restrictive configurations leading to increased cadherin clustering. As Fer-mediated tyrosine phosphorylation of cortactin and gelsolin association are transient events limited to nascent contact assembly, they may coordinate the remodeling of actin networks that facilitate AJ maturation (Fig. 2).

5.4. Regulation of actin binding proteins through PIP2

The formation of N-cadherin adhesions may be regulated through association with lipid rafts [84]. These plasma membrane domains can stabilize N-cadherin junctions at regions of intercellular contact [84]. Although the biological mediators within these rafts is not defined, it is likely that the phosphoinositide, phosphatidylinositol 4, 5 bisphosphate (PIP2) plays crucial roles. PIP2 is a well-characterized phosphoinositide and is an important regulator of several actin binding proteins (reviewed in [85]). Consistent with this view, actin assembly dynamics are tightly associated with N-cadherin junctions and lipid rafts [84].

PIP2 has been shown to be generated at sites of N-cadherin ligation in a PIP5KIγ dependent mechanism (our unpublished findings). N-cadherin mediated RhoA activation is necessary for the recruitment of PIP5KIγ to sites of N-cadherin ligation (Fig. 2: early N-cadherin ligation). While the mechanistic details involved in RhoA mediated PIP5KIγ recruitment are still unknown, ROCK signaling may play an important role. Indeed GDP-RhoA has been suggested to bind constitutively to PIP5KIγ, shuttling the molecule to distinct subcellular locations where subsequent RhoA activation, and associated ROCK signaling displace the bound PIP5KIγ [86]. As ROCK activation has been detected at sites of cadherin ligation [71], PIP5KIγ delivery to sites of N-cadherin ligation may be regulated by localized Rho-ROCK signaling events (Fig. 2).

PIP5KI generated PIP2 inhibits gelsolin severing, stimulates uncapping [87,88] and thereby contributes to the generation of large numbers of polymerization-competent barbed ends. This facilitates for the rapid remodeling of local actin networks. As PIP2 is an important regulator of many actin binding and regulatory proteins [85], it is likely to be of central importance in the regulation of actin remodeling events at sites of N-cadherin ligation (Fig. 3). The dynamic remodeling of actin networks at nascent contacts may occur in conjunction with cortactin tyrosine phosphorylation. Together, these molecules may facilitate the increased mobility and clustering of N-cadherin molecules at contact regions.

The maturation and increased adhesion strength of contacts is a function of lateral clustering and actin remodeling at regions peripheral to the central adhesion zone; the contact zone is then extended (Fig. 3). These peripheral areas may be "microniches" that replicate the events found at initial contacts. An increased area or band of adhesion may enhance resistance to separation and promote the intercellular adhesive strength of maturing contacts. While recent evidence indicates that the cadherin adhesion contacts are not statically linked to subcortical actin filaments [89], continued remodeling of this network may be necessary for intercellular contact formation and zone extension [75].

6. Conclusions

The formation and regulation of cadherin-mediated AJs in fibrous connective tissues has received little attention despite broad ranging biological and clinical significance. N-cadherin-mediated AJs exhibit distinct biological behavior and markedly different tissue-specific expression profiles. More detailed characterization of the mechanisms that regulate the formation and remodeling of N-cadherin adhesions will illuminate basic processes in wound healing and malignant transformation in connective tissues.

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